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The Patterns of Bromodeoxyuridine Incorporation in the Nervous System of a Larval Ascidian, *Ciona intestinalis*

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Abstract. The fates of cells from the anterior region of the ascidian neural plate are described either as neural or as mixed neural and non-neural. In *Ciona intestinalis*, all cellular progeny are accounted for until a time 60% between the onset of embryonic development and larval hatching. To resolve the issue of their fates in this species, we have examined the later mitotic history of neural-plate cells. Because cessation of cell division in the neural plate has been claimed to occur at 70% of embryonic development, we need to account for cell production from 60% onward, to determine whether more cells are produced than populate the larval CNS, allowing some to adopt non-neural fates. The embryonic incorporation of bromodeoxyuridine (BrdU), 500 μM in seawater, was monitored in 1-h larvae by anti-BrdU immunocytochemistry. The pattern of incorporations indicates that all larval neurons are born before 70% of embryonic development, but that cell division unexpectedly continues to generate ependymal cells until at least 95%. Divisions in the neurohypophysis continue throughout embryonic development. The total number of cells produced appears sufficient only to complete the complement of larval CNS cells, denying non-neural fates for anteriorly migrating neural plate cells, and indicating a general absence of cell death. Consistent numbers of incorporations after the same exposure in different larvae provide evidence for determinacy of neural plate lineages. The last three conclusions confirm those reached previously (Nicol and Meinertzhagen, 1988b).

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Introduction

Ascidians or sea-squirts have long attracted interest because, as urochordates, they are considered to be closely related to the common ancestor of all chordates (Garstang, 1928; Berrill, 1955; Bone, 1972). It is not the sessile adult, however, but rather the short-lived larval stage that exhibits some of the typical features of a chordate. These include a dorsal tubular central nervous system (CNS), and the fact that this arises from an embryonic neural plate. On the other hand, the number of cells within the larval CNS is approximately 370 and is apparently fixed, at least within narrow limits, in closely related offspring of the mutual fertilization of two hermaphrodite adults (Nicol and Meinertzhagen, 1991). Such cell constancy, or eutely, is not an invariably identifiable feature of chordate nervous systems (Williams and Herrup, 1988), but rather is usually associated with invertebrate animals. Most of the cells constitute the functional larval nervous system, which consists of an anterior sensory vesicle, the visceral ganglion in the posterior part of the trunk, and the nerve cord in the tail (Nicol and Meinertzhagen, 1991; Fig. 1). The formation of this CNS follows the pattern in other chordate embryos (Nicol and Meinertzhagen, 1988a), with neurulation following the formation of the neural plate.

Two current ideas exist about the fate of the cells in the embryonic neural plate. Descriptive studies in *Ciona* (Nicol and Meinertzhagen, 1988a,b) indicate that all neural plate progeny contribute to the CNS, and thus offer an orthodox view of the fate of this structure in ascidians. On the other hand, injections of horseradish peroxidase (HRP) into seventh and eighth generation neural-plate blastomeres in *Halocynthia* (Nishida and Satoh, 1985; Nishida, 1987) imply that some anterior neural plate cells

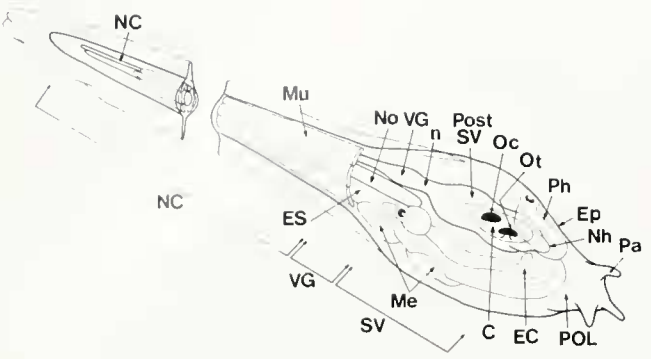

Figure 1. Diagram of the entire larva, from the right rostral side. Abbreviations: Nervous system. C: cavity of the sensory vesicle; n: neck; NC: nerve cord; Nh: neurohypophysis; Oc: ocellus; Ot: otolith; PostSV: posterior sensory vesicle; SV: sensory vesicle; VG: visceral ganglion. Other systems: EC: endodermal cavity; Ep: epithelium; ES: endodermal strand; Me: mesenchyme; Mu: muscle band; No: notochord; Pa: papilla; Ph: pharynx; POL: pre-oral lobe. (From Nicol and Meinertzhagen, 1991.)

migrate anteriorly, away from the CNS, where they acquire non-neural fates either in the pharyngeal region or in the epidermal adhesive papillae. If migration of neural plate cells were also to occur in *Ciona*, it must, according to the results of Nicol and Meinertzhagen (1988b), occur later than the 60.5% developmental stage (where 100% is the period of embryonic development required to attain larval hatching), because this was the last record of these workers. If migration were to occur, it would further require that extra cell divisions occur to replace the migrating cells (Nicol and Meinertzhagen, 1988b). The resolution of this issue hinges on an accurate determination of the time at which the last cell divisions take place.

When does cell division actually cease? Reliable information about the actual time for cessation of cell divisions in the CNS is lacking. Berrill (1935) suggests, but without clear documentation, that the onset of pigment differentiation in the ocellus and otolith (70% of embryonic development) marks the end of neural proliferation, and this claim was used to arbitrate the differences between neural-plate fates in *Ciona* and *Halocynthia* (Nicol and Meinertzhagen, 1988b). Cell counts in *Ciona*, however, indicate that this is not accurately the case (Fig. 2). At 70%, some 300 cells exist in the CNS (Nicol, 1987), while at hatching there are 371 or so, implying that further proliferation must occur between 70–100%. Previous cell counts have indicated that 88% of the final cell complement in one larva was attained by about 73% (Balinsky, 1931), and thus confirm this picture. Our study was designed to address the question of whether later mitoses do actually occur in the neural plate, as these figures suggest. If these mitoses do occur, then our aim was to identify the location, if not the fate, of the resultant progeny. To do this, we have exploited the incorporation of a substituted nucleotide, 5-bromodeoxyuridine (BrdU), to expose late cell divisions in the neural tube. Our observations build on the only precedent in this direction (Reverberi *et al.*, 1960), a previous study in which thymidine incorporations were examined, but only at earlier stages in neurulation.

Materials and Methods

Adult *Ciona intestinalis* were obtained from the Marine Resources Department of the Marine Biological Laboratory, Woods Hole, Massachusetts, or from Tjärnö Marine Biological Laboratory, Sweden, and maintained in running seawater. Gametes collected from the gonoducts of two individuals were mixed to produce cross-fertilized embryos, which were then raised in plastic petri dishes floating on a water bath at 16°C.

DNA synthesis was monitored by the incorporation of BrdU (Sigma), and revealed by a monoclonal antibody directed against BrdU (Gratzner, 1982). Incorporations occurred when embryos were immersed in BrdU dissolved in Millipore-filtered seawater. Initially, three concentrations, 250 μM, 500 μM, and 1 mM, were tested. The lowest concentration produced less clear immuno-staining. Although the two higher concentrations both gave equally strong immuno-labeling, the lower of the two, 500 μM, was chosen. It exceeded the detection threshold for

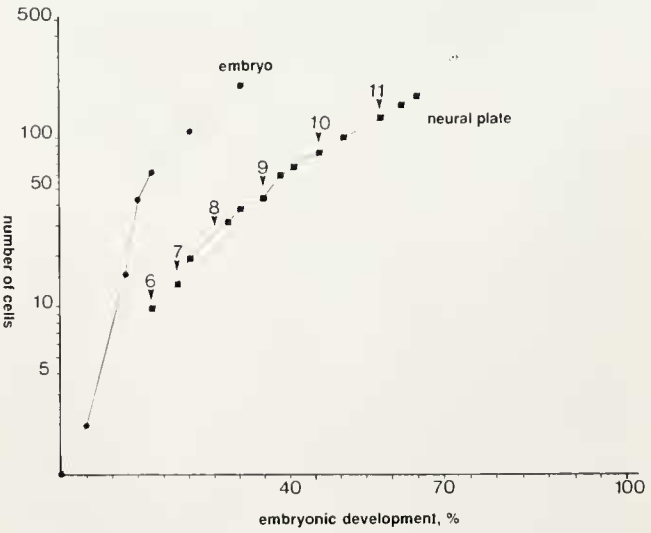

Figure 2. Cell proliferation at different stages in the neural plate revealed from cell counts (ordinate: square symbols, data plotted from Nicol and Meinertzhagen, 1988a, b), compared with cell number in the entire embryo (circles, data from Conklin, 1905; Nicol and Meinertzhagen 1988a). Arrowheads indicate the generation number of the cells at that stage. The increase in the number of neural plate cells is roughly exponential, as shown by their linear progression in this plot, and attains the cell complement of the swimming larva (100%, open square, data from Nicol and Meinertzhagen, 1991) only at a time projected to occur later than 70% (open square, data from Nicol, 1987).

dividing cells in adult *Ciona* (Bollner *et al.*, 1991), but avoided an unnecessarily high concentration, which could have disturbed embryonic development. This type of perturbation has been previously reported for *Ciona* (Cusimano, 1961) and is also widely known in other embryos, such as, for example, insects (Truman and Bate, 1988).

Two strategies for BrdU incorporation were used (Fig. 3). In the first, incorporations resulting from long-pulse exposures to BrdU which started at 60% of embryonic development or later, and ended when the larva hatched, were used to reveal all cells that were not already postmitotic before these times. Long-pulse exposures were started at 60%, 70%, 72.5%, 75%, and so on, and were extended in 2.5% increments up to 95%. In the second strategy, short-pulse BrdU incorporations of 30 min (about 2.5%) were also administered, starting at 70% and then at the same intervals as for the long pulses, but with the embryos transferred to fresh seawater after the pulse. Short-pulse incorporations were used to reveal the regional and temporal distribution of mitoses. In all cases, larvae were fixed 1 h after hatching.

Fixation for pre-embedding immunocytochemistry was carried out in 0.1 *M* phosphate buffer (pH 7.2) containing 4% paraformaldehyde, for 4–12 h at 4°C. After fixation, the specimens were washed in the same buffer and treated with 2 *N* HCl in phosphate-buffered saline (PBS) for 45 min, and then rinsed in PBS (pH 7.2) containing 0.1% Triton X-100 (Sigma) (PBS-TX). To improve tissue penetration of antibodies, the fixed larvae were treated with 1% Triton X-100 PBS for 24 h at 4°C. After two washes in PBS-TX, samples were incubated for 48 h at 4°C in anti-BrdU (Becton and Dickinson, Mountain View, California) diluted 1:200 in PBS-TX with 0.5% bovine serum albumin (BSA). The preparations were incubated overnight at 4°C in rabbit anti-mouse secondary antibody (Dakopatts) diluted 1:50 in PBS-TX BSA, followed by mouse PAP-complex (Dakopatts) diluted 1:100 in PBS-TX BSA, before further processing using 0.03% DAB as a chromogen. The preparations were washed in PBS-TX between all steps. Finally, the material was dehydrated and embedded, either in Historesin (LKB) or in Durcupan ACM (Fluka), viewed either as wholemounts or as 1–3-μm sections cut on glass knives, and photographed with differential interference contrast optics.

For post-embedding immunocytochemistry, specimens were fixed either in methanol for 20 min at −20°C, followed by ethanol under the same conditions, or in cold Bouin's fluid for a minimum of 3 h to a maximum of overnight. All specimens were stained with eosin dissolved in 70% alcohol, dehydrated in a routine ethanol series, and embedded in paraffin wax. Sections were cut either transversely or sagittally at 4–5 μm and mounted on poly L-lysine coated slides. The preparations were then dewaxed, rehydrated, and treated with 2 *N* HCl for 30 min, prior to overnight incubation in anti-BrdU 1:200. The same secondary antiserum and PAP-complex were used as for the wholemounts, and preparations were incubated for 1 h and 30 min, respectively. The DAB reaction was enhanced by adding $NiCl_2$ to a final concentration of 0.015%.

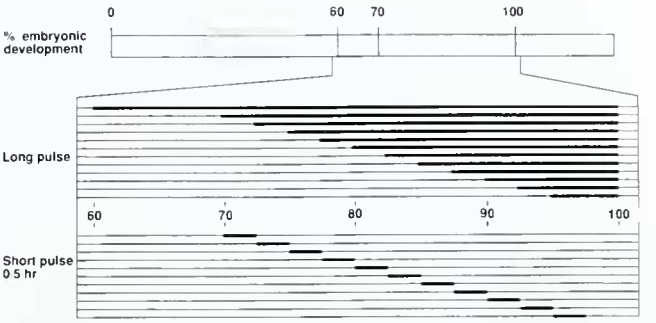

Figure 3. Diagrammatic representation of the two types of BrdU exposure used. Long-pulse exposures (heavy lines) commence during the final 40% of embryonic development and continue until hatching of the larva. Short-pulse exposures (heavy lines) distributed throughout the same period, but in abutting intervals of 30 min.

Results

The larval ascidian CNS is divided into three main regions (Fig. 1), which, in sequence, consist of the anterior sensory vesicle, with 215 cells, separated by a neck region of 6 cells from the visceral ganglion of 45 cells, which lies in the posterior part of the trunk, and the nerve cord of 65–66 cells in the tail. The sensory vesicle is situated rostrally in the trunk with its anterior left portion overlain by the pharynx and the so-called neurohypophysis, the primordium of the adult neural complex with some 40 or so cells. These cell numbers derive mostly from one larva (L4) in Nicol and Meinertzhagen (1991), but are supported by others reported by these authors and by Nicol (1987). Only some cells are neuronal. Roughly 68% have been classified as ependymal, a specialized non-neuronal cell peculiar to embryonic and larval chordates, from their position lining the cavities of the neural tube's elaborations or from clear similarities in the cytological appearance to those that do (Nicol and Meinertzhagen, 1991).

Initially, long pulse exposures were used to establish how late in embryonic development cells of the CNS incorporated BrdU. Larvae were examined in wholemounts, after being immersed in BrdU from 70% of embryonic development and allowed to develop in this medium until they hatched as free-swimming larvae (Fig. 3). Embryos of the correct developmental stage were selected according to the time since their fertilization expressed as a proportion of the time until hatching, which for most batches was attained after 20 h at 16°C. In addition, the devel-

opment of a batch of embryos was calibrated by two further criteria. The first was that 60% of embryonic development has occurred when the length of the tail bud equals that of the trunk, and the second was that 70% has occurred when pigment cells first appear in the sensory vesicle.

Long pulse BrdU exposures starting at 70% of embryonic development produced labeled nuclei in all parts of the CNS within the trunk, but not in the nerve cord (Fig. 4a). It is thus immediately clear that mitotic activity in the CNS extends beyond 70%. With a single short BrdU pulse administered from 72.5% to 75%, labeled nuclei were detected in both the dorsal part of the sensory vesicle and the neurohypophysis (Fig. 4b). A long BrdU pulse starting from 85% and continuing to 100% (Fig. 4c) gave labeled nuclei in the sensory vesicle as well as in the neurohypophysis, while a short BrdU pulse starting from the same stage, 85%, produced labeled nuclei in the ependymal lining of the nerve cord and in the neurohypophysis (Fig. 4d). Thus the cells in the sensory vesicle must have had their S-phase after the period of the short pulse, *i.e.*, later than 87.5%.

BrdU exposure still produced labeled nuclei in the CNS at 95% of embryonic development. At this late stage, a few labeled nuclei were found in, or in close association with, the caudal part of the sensory vesicle or the neck (Figs. 4e, 5). These cells may have been part of the ependymal lining of the nerve cord. Mitotic activity was also evident in the neurohypophysis at this stage (Figs. 4e, 5).

Some idea of the extent of mitotic activity can be gained from the numbers of labeled nuclei. Counts in the CNS of long-pulse larvae, which were incubated in BrdU starting at 60, 70, or 85% and continued until hatching, are shown in Table I.

When the long BrdU pulse started at 60% embryonic development approximately 50 nuclei (53, 50) showed staining in the larval CNS, excluding the neurohypophysis. These included cells in the sensory vesicle, the neck, and the visceral ganglion. Some of the labeled cells in the sensory vesicle were deemed to be neurons from their location, whereas all other labeled cells at this stage were thought to be ependymal (Fig. 6a). Most labeled cells in the visceral ganglion were members of bilateral pairs forming the ependymal lining of the nerve cord.

When the long BrdU pulse started later, at 70% embryonic development, fewer labeled nuclei appeared in the CNS (31, 34 cells: Table I), most of which were in the sensory vesicle (Fig. 6b). The larva with 31 labeled nuclei had only 6 that could be assigned to the visceral ganglion. On the other hand, the second larva possibly had eight incorporations in its visceral ganglion, but because the visceral ganglion and surrounding mesenchymal cells were difficult to distinguish in this larva, some of the eight may not have been part of the CNS. In both larvae, some labeled cells in the posterior part of the sensory vesicle and others in the anterior visceral ganglion might have been neck cells, but this could not be ascertained.

Very few cells were labeled from long-pulse BrdU exposures starting at 85% of embryonic development and extending until hatching (Table I). In one case, seven labeled cells were found in the CNS, while in the second case, the nine cells in the CNS were found in the sensory vesicle and none was found in the visceral ganglion. Of the nine, at least five could be assigned as ependymal cells lining the nerve cord. The identity of the remaining four was unclear, although they were found close to the wall of the sensory vesicle (Fig. 6c), an area where most of the cells are also thought to be ependymal (Nicol and Meinertzhagen, 1991).

These results are summarized in Figure 7. In all the examined groups, BrdU incorporations were detected in the neurohypophysis, which thus seemed to continue to proliferate throughout the entire period investigated.

Discussion

The primary conclusion of this study is that far from ceasing at the time of pigment differentiation at 70%, as Berrill (1935) has suggested, cell division in the nervous system continues throughout the remaining period of embryonic development. In principle, therefore, cells could arise in excess of those surviving in the CNS, and this excess could allow for some to migrate anteriorly, as current evidence indicates must happen in *Halocynthia* (Nishida, 1987). The production of surplus cells had previously been thought unlikely because the progressive increase in the duration of the cell cycle seemed to preclude the possibility for there to be sufficient time for additional rounds of cell division before 70% (Nicol and Meinertzhagen, 1988b).

Is there in fact sufficient time to generate an excess of cells? The observed patterns of divisions within the neural plate concluded with 84 progeny in the posterior region of the neural plate and 85 in the anterior at 60.5% (Nicol and Meinertzhagen, 1988b). Nicol (1987) predicted from the pattern of their previous divisions that these cells will soon generate a total of 109 11th and 12th generation cells in the posterior part of the neural plate, and 158 such cells in the anterior (of which only the pigment cells are still in their 9th generation). She predicted that these would occur before 70%, although some additional divisions must also be required to make up the total number of 300 cells counted at that stage (Nicol, 1987). This means that 130 divisions are required between 60% and 70%, an interval corresponding to 2 h at 16°C. Most of the 170 neural plate cells recorded at 60.5% (Nicol and Meinertzhagen, 1988b: their 13.3 h) were already born 48 min earlier at 57% which, considering a cell cycle time

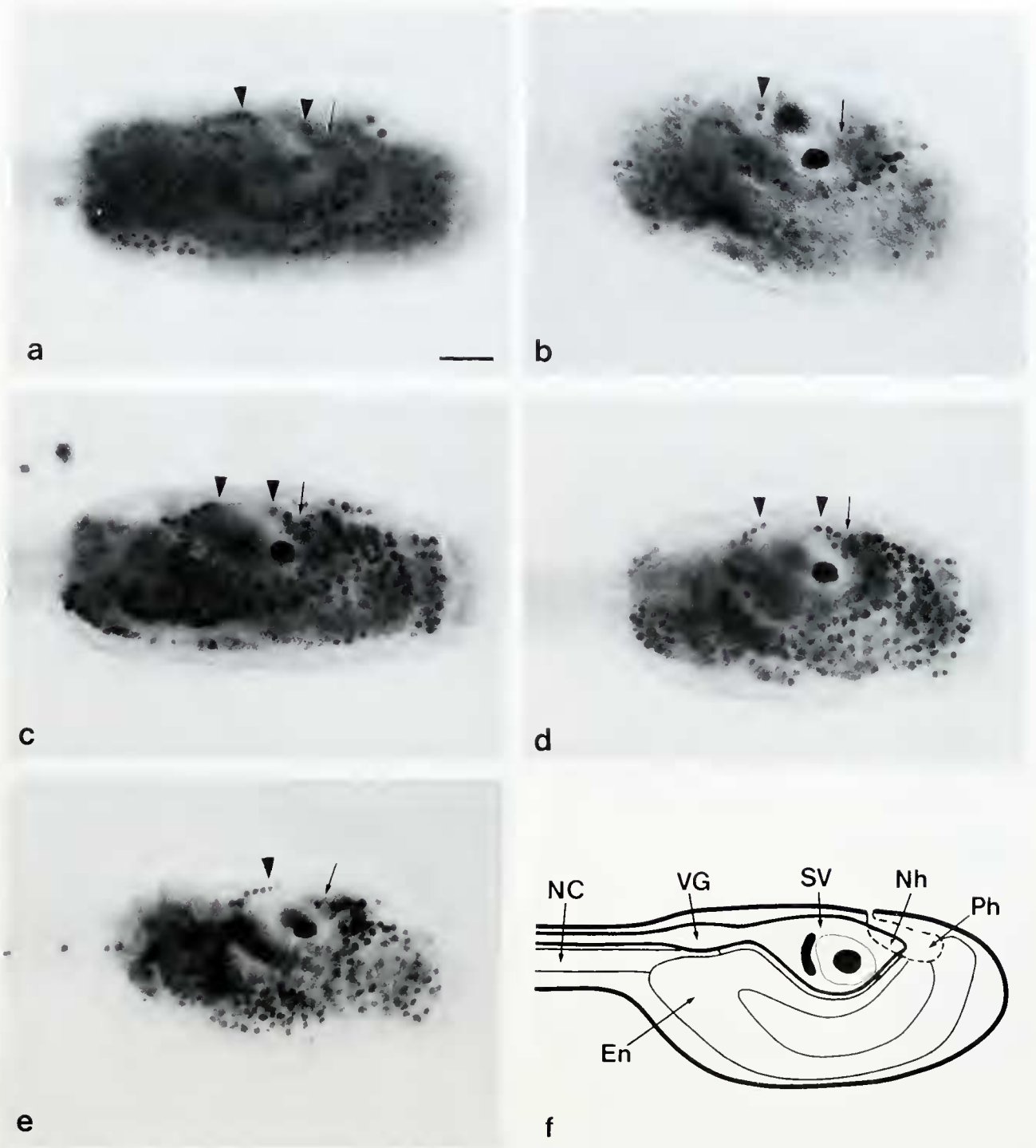

Figure 4. Larval wholemount preparations showing the pattern of incorporations when the embryo is exposed to the BrdU label at different ages. Incorporations occur in nuclei in the sensory vesicle (arrowheads) and neurohypophysis (arrows); scale bar: 30 μm. (a) Long-pulse embryo exposed through the period from 70% to hatching. Labeled nuclei appear in the dorsal and caudal parts of the sensory vesicle, in cells associated with the neck between the sensory vesicle and visceral ganglion, and in the neurohypophysis. (b) A short BrdU pulse at 72.5% (Fig. 3B) labeled nuclei in the posterior part of the sensory vesicle and the neurohypophysis (out of focus). (c) A long BrdU pulse starting at 85% labeled nuclei in the posterior part of the sensory vesicle, along the nerve cord, and in the neurohypophysis. (d) A single BrdU pulse at 85% of embryonic development gave labeled nuclei in the neurohypophysis and along the dorsal nerve cord. (e) A single pulse at 95% incorporated in nuclei of the dorsal posterior sensory vesicle. (f) Diagram of the trunk shown with the same orientation as (a–e), identifying the positions of the major components: endoderm (En), neurohypophysis (Nh), pharynx (Ph), and sensory vesicle (SV).

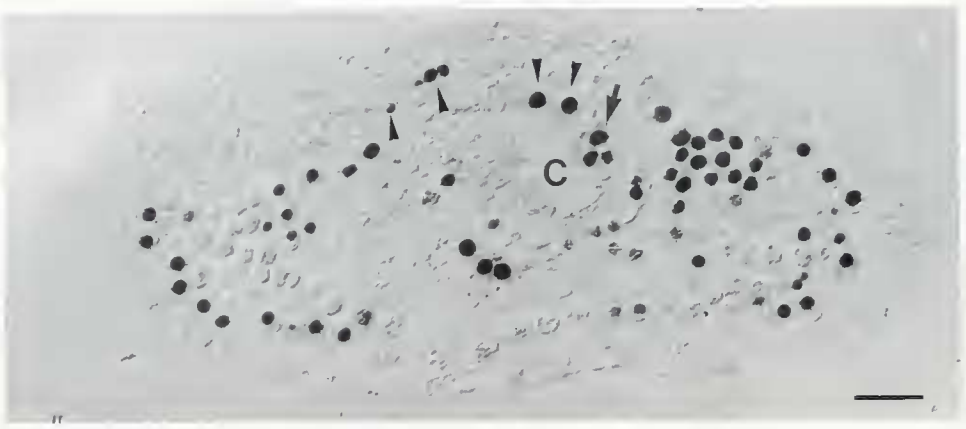

Figure 5. A single BrdU pulse at 95% showing labeled nuclei abutting the cavity (C) of the sensory vesicle, in cells of the caudal part of the sensory vesicle (arrowheads), as well as in the neurohypophysis (arrows) of a wholemount section. Bar = 20 μm.

of 2.5–2.9 h (Nicol and Meinertzhagen, 1988b), gives sufficient time for the 130 divisions needed to reach a total of 300 cells by 70% (15.3 h).

The numbers of cells predicted by Nicol (1987) compare closely in the posterior part of the neural plate with those found in the final larva (65–66 cells in the nerve cord + 45 cells in the visceral ganglion: Nicol and Meinertzhagen, 1991). On the other hand, for the anterior part where 221 cells exist in the larva (266 trunk cells–45 cells in the visceral ganglion of larva L4, in Nicol and Meinertzhagen, 1991), 63 cells (=221–158) would have to divide again just to generate the numbers of larval CNS cells, without excess. Other larvae may have slightly differing cell counts. The number of extra divisions for the four larvae in Nicol and Meinertzhagen (1991) would, for example, range from 56 to 65. These numbers, moreover, take no account of the neurohypophysis, which requires approximately another 40 cell divisions, for a total of 103 divisions.

Are more than 103 BrdU incorporations in fact seen in the period between 60% and hatching, for any excess cells to be created? The 53 or so labeled cells that have been counted in the larval CNS from long-pulse exposure at 60% would seem quite clearly to indicate not an excess of divisions, but an insufficiency. On the other hand, if Nicol's (1987) record of 300 CNS cells at 70% is correct, we can account for the remaining divisions: approximately 30 cells are born from 70% to hatching (Table I) which together with 40 or so cells in the neurohypophysis produces a total of 370. The inconsistency between our data and the predictions arising from Nicol's data (Nicol, 1987) stems, then, from the small number of BrdU incorporations seen in our long-pulse animals at 60%.

There are several possible reasons for these small numbers. (1) The staging of our embryos might have differed from those of Nicol, so that the BrdU pulses actually commenced later than 60%. Since many of the neural plate cells at this stage had been in their 11th generation for some time, it is not impossible that they were about to enter their next generation at about the 60% stage. Fewer incorporations than expected could then have resulted if the pulse were, through slightly inconsistent staging of embryos in the two studies, administered a little later than 60%. The fact that incorporations counted from larvae exposed at 60% derived from a different batch of animals than those used to count incorporations in older larvae (Table I) provides a possible explanation for the internal discrepancy in our study between the 60% batch and the 70 and 85% batches if, once again, the youngest batch had in fact actually been somewhat older than 60%.

(2) We also do not know the details of the cell-cycle. It is therefore possible that although cells were pre-mitotic, they had already passed, or were at least in a late part of their S-phase and were consequently unable to incorporate sufficient BrdU to be detected.

(3) A third possibility is that embryonic development was slightly inhibited from the 60% stage by the concen-

Table I

Number of CNS cells labeled with BrdU[1]

Stage	Larva 1	Larva 2
60–100%	53	50
70–100%	31	34
85–100%	9	7

[1] Two larvae counted at each stage. The older larvae were all from the same two adults and were processed together, while those labeled from 60% were larvae of two adults which received an identical BrdU treatment, but on a different day.

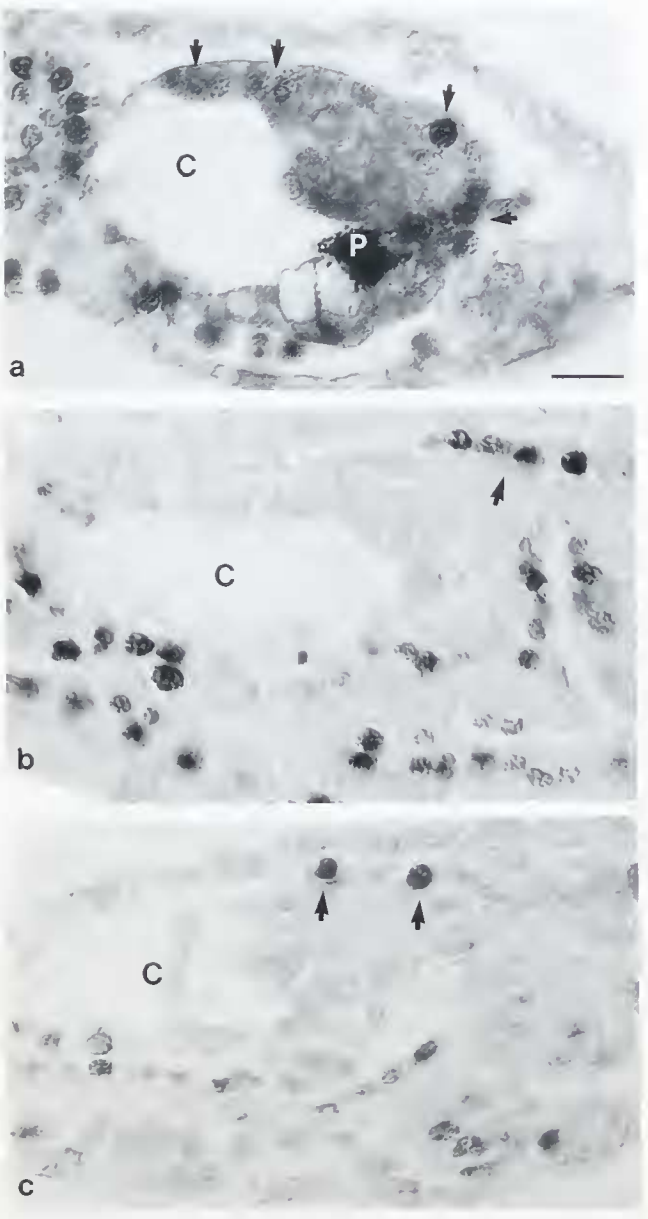

Figure 6. Sections through the sensory vesicle of long-pulse larvae exposed to BrdU from 60 to 85%. (a) 60%. Incorporations occur in the sensory vesicle wall and a group posterior to the pigment cup. (b) 70%. Incorporations occur in ependymal cells lining the dorsal nerve cord. (c) 85%. Incorporations occur in two labeled nuclei in the ependymal lining of the nerve cord. Positions of labeled nuclei are indicated by arrows. C: sensory vesicle cavity; P: pigment cup. Bar = 10 μm.

tration of BrdU we used. Cusimano (1961) reports that after exposing embryos to 500 μ*M* deoxuridine from early cleavage stages onward, 90% of normal larvae emerged, whereas exposure to 1 m*M* was clearly toxic. Equivalent data to these are lacking for BrdU, which we suggest has no effect on normal development at 500 μ*M*. Not only were the periods for which our embryos were immersed in BrdU much shorter than in Cusimano's study, but all the resultant larvae arising from such treatment also looked normal. It is nevertheless possible that their cell division had been slowed or inhibited by long-pulse exposure to BrdU, but that this was undetectable externally, and that as a result the number of incorporations was smaller than the normal number of divisions. In that case, the effect upon embryos at 70% might be less than at 60% because of the shorter cumulative period of exposure and because the susceptibility of embryos starts at the neurula stage (Cusimano, 1961). We were not able to avoid the possibility of this problem by using BrdU at a lower concentration (250 μ*M*), because that would have diminished the staining intensity and run the risk of recording too few incorporations.

Thus, with these provisos, the evidence supports a mode of neural plate proliferation which at most attains constancy of cell number by addition (Williams and Herrup, 1988), possibly by a lineage dependent mechanism. This proliferation continues, moreover, until the final stage of embryonic development, because the latest stages investigated (95%) still revealed incorporations in the CNS. The cells produced at this late stage are thought to be ependymal rather than neuronal, as indeed is true for most

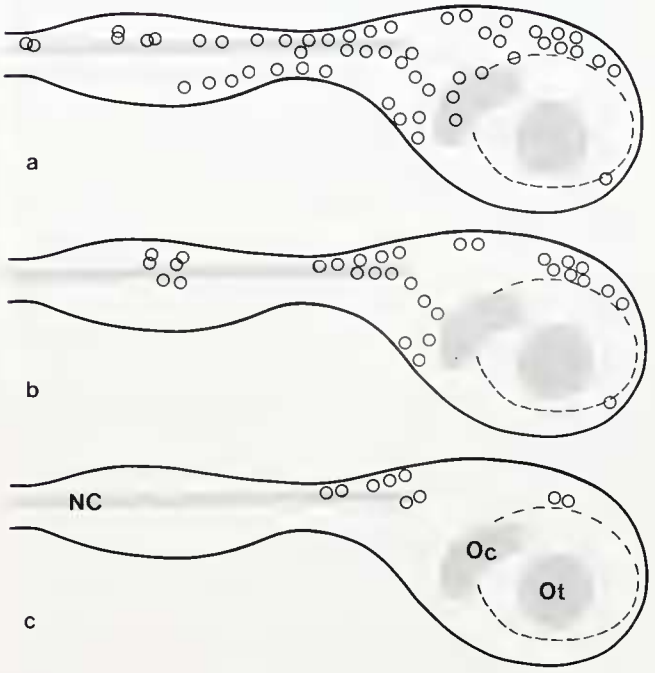

Figure 7. Schematic representation of the positions of labeled nuclei in three counted stages, made by superimposing *camera lucida* drawings. (a) Long-pulse larva labeled at 60%. (b) Long-pulse larva labeled at 70%. (c) Long-pulse larva labeled at 85%. Nuclei are shown with equal sizes; compare their positions with maps of neuronal nuclei (Nicol and Meinertzhagen, 1991: their Figs. 16, 17). Ot: otolith; Oc: ocellus; NC: nerve cord.

BrdU labeled nuclei found in the larval CNS after 60%. This diagnosis is based on the locations of the nuclei relative to published maps (Nicol and Meinertzhagen, 1991), rather than the appearance of the nuclei themselves, which did not distinguish ependymal cells from neurons. Our data thus indicate that all neurons in the larval CNS are born before 70% of embryonic development, and that only ependymal cell incorporations are revealed thereafter, at least outside the neurohypophysis. The neurohypophysis itself continues to proliferate right throughout the period of embryonic development, and thus behaves differently from the cells of the larval CNS proper.

What happens to the cells produced from divisions between 70 and 100% of embryonic development? Three possibilities concerning their fate exist in the light of our new evidence.

The first and by far most likely possibility is that the late divisions of neural plate cells are the final rounds required for the constructive generation of the cells of the CNS by a mechanism of addition (Williams and Herrup, 1988), topping up the complement of neural plate cells to the final total, which, including roughly 40 cells in the neurohypophysis, is about 370 (Nicol and Meinertzhagen, 1991). The only way in which a fixed number of larval CNS cells could then be produced would be by regulating the number of progeny of each neural plate cell, since the total number of such cells in the neural plate is fixed (Nicol and Meinertzhagen, 1988b). If, on the other hand, a small surplus of cells were produced, if for example we were unable to record all incorporations successfully, two further possibilities on the fate of the cells can be entertained.

First, they may migrate away from the neural plate. The late migration of cells to other regions is implied by Nishida's (1987) results on *Halocynthia*, but because incorporations were not found in the adhesive papillae in *Ciona*, and because this was a clear site of their localization in *Halocynthia* (Nishida, 1987), these two species must differ in at least this one important respect, reinforcing the only conclusion reached previously on their comparison (Nicol and Meinertzhagen, 1988b). Migration to sites other than the papillae is at least theoretically possible. For example, we see pharyngeal incorporations, as Nishida (1987) found in *Halocynthia*, but these need not be of migrating cells. Moreover, from BrdU immunocytochemistry we cannot distinguish them from incorporations in the neurohypophysis, because the neurohypophysis is located close to the pharynx and we lack probes to its yet undifferentiated cells.

Finally, surplus cells may be later eliminated by cell death. This has been ruled out for the early stages of neurogenesis (Nicol and Meinertzhagen, 1988b) and is most unlikely for later stages. At least in the nerve cord of the tail, cell death apparently does not occur. Cell complement at 60% has already attained that of the mature larva (Nicol and Meinertzhagen, 1988b, 1991) and we find no BrdU incorporations at this site, so that all postmitotic cells must survive into the larva. To rule out cell death may seem a trivial distinction in the case of the nerve cord, however, given that its cells will in any case degenerate with the onset of metamorphosis, soon after larval hatching (Cloney, 1978), and because probably all such cells are ependymal not neuronal. Nevertheless, the absence of cell death is a cardinal difference seen when we invoke the other major example of chordate neurogenesis, the formation of the CNS in vertebrates (Oppenheim, 1991).

We are therefore confident that our results support all of the following: the absence of migration to the papillae from the neural plate, the birth of all neurons before 70% but continued ependymal cell production until at least 95%, the lack of over-production of neural plate progeny and the consequent absence of neuronal cell death, and the attainment of final cell number by the regulated control of the number of cell divisions. Evidence for the latter comes clearly from consistency among the numbers of incorporations seen in larvae after exposure to BrdU at a particular embryonic stage. Thus, larvae exposed at the same times between 60 and 100% differ by only two to three incorporations (Table I). We take the small range of the differences between two animals at any one larval age to reflect either minor differences in the staging of the larvae or in the timing of their mitoses, and to indicate a determinacy of cell lineage amongst neural plate cells which has been seen previously (Nicol and Meinertzhagen, 1988b). Put another way, if we assume the existence of lineage determinacy, consistency in the number of BrdU incorporations provides assurance that developmental staging and immunocytochemical detection is also consistent between embryos.

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